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(54) Title: DIVALENT ANTIBODY FRAGMENTS

(57) Abstract

Divalent antibody fragments are described, each of which has one or more interchain bridges containing a synthetic or naturally occurring polymer selected from a polyalkylene, polyalkenylene, polyoxyalkylene or polysaccharide. Each bridge may be the residue of a homo- or heterobifunctional cross-linking reagent and serves to link two heavy chains in each antibody fragment via the sulphur atoms of cysteine residues present in the chains. Each fragment may be attached to one or more effector or reporter molecules, and is of use in therapy or diagnostics where it has markedly improved binding and/or pharmacokinetic properties when compared to other antibody fragments which have the same number and type of polymer molecules but in which the polymer molecules are randomly attached.

Non-Reducing
1. Molecular weight marker proteins
2. Fab' and DFM mix
3. Purified DFM-PEG (10kDa SS linker)
4. Purified DFM-PEG (20kDa SS linker)
5. Purified DFM-PEG (40kDa)

Reducing
6. Fab' and DFM mix
7. Purified DFM-PEG (10kDa SS linker)
8. Purified DFM-PEG (20kDa SS linker)
9. Purified DFM-PEG (40kDa)

DIVALENT ANTIBODY FRAGMENTS

This invention relates to modified divalent antibody fragments, to processes for their preparation, to compositions containing them and to their use in medicine.

Antibodies are increasingly being used in the clinic for diagnostic and therapeutic purposes. The aim in each case is to exploit the combination of high specificity and affinity of the antibody-antigen interaction, to enable detection and/or treatment of a particular lesion. The antibody is used alone, or is loaded with another atom or molecule such as a radioisotope or cytotoxic drug.

The pharmacokinetics and biodistribution of an antibody play a major role in determining whether its use in the clinic will be successful. Thus the antibody must be capable of being delivered to the site of action and be retained there for a length of time suitable to achieve its purpose. It also should be present only at sub-toxic levels outside of the target and it must be catabolised in a well-defined manner.

For many uses the pharmacokinetics of antibodies are not ideal. This is especially true for tumour diagnosis and therapy with antibody-radioisotope or drug conjugates. For diagnosis with such conjugates long half-lives limit the tumour-to-background ratio and hence the sensitivity of lesion detection. For therapy, a long half-life leads to long-term exposure of normal tissues to the antibody conjugate and hence to dose-limiting toxicity.

A number of approaches are available to manipulate the pharmacokinetics of antibodies, and these usually also affect their biodistribution. The simplest and most generally applicable approach is the use of antibody fragments. These are cleared more rapidly from the circulation than whole antibodies and distribute more rapidly from the blood to the tissues, which is a particular advantage in some applications, for example for tumour imaging and therapy.

Previous studies with antibodies and antibody fragments have used random PEG attachment via lysine residues [e.g. Ling, T. G. I. & Mattiasson, B. J. Immunol. Methods (1983), 59, 327-337; Wilkinson, I. *et al* Immunol. Letters (1987) 15, 17-22; Kitamura, K. *et al* Cancer Res. (1991), 51, 4310-4315; Delgado, C. *et al* Br. J. Cancer (1996), 73, 175-182] and thiolated derivatives [Pedley, R. B. *et al* Br. J. Cancer (1994), 70, 1126-1130]. Random attachment has often resulted in modified antibodies which are only able to bind their target antigen with reduced affinity, avidity or specificity. In one attempt to overcome this, critical lysine residues in antigen binding (CDR) loops have been replaced with arginines to allow modification with less loss in immunoreactivity [Benhar, I. *et al* Bioconjugate Chemistry (1994) 5, 321-326].

Specific sites in the constant and the hinge regions of antibodies can be engineered to allow site-specific linkage of a range of effector and reporter molecules [Lyons, A. *et al* Prot. Eng. (1990), 3, 703-709; and European Patent Specifications Nos. 348442 and 347433]. We have now determined that site-specific attachment of polymers to divalent antibody fragments can be used to avoid the loss of immunoreactivity previously associated with random attachment processes. Furthermore, fragments modified in this way have markedly improved binding and/or pharmacokinetic properties when compared to fragments which have been modified randomly with the same number and type of polymer molecules.

Thus according to one aspect of the invention we provide a divalent antibody fragment comprising two antibody heavy chains and at least one polymer molecule in covalent linkage, each heavy chain being covalently linked to the other by at least one non-disulphide interchain bridge linking the sulphur atom of a cysteine residue in one chain to the sulphur atom of a cysteine residue in the other chain, said cysteine residues being located outside of the variable region domain of each chain, characterised in that at least one non-disulphide interchain bridge contains a covalently linked polymer molecule.

The term "non-disulphide" as used herein is intended to mean that S-S bridges, e.g. of the type normally found in antibodies, are excluded. An

In general each heavy chain and, when present, light chain, will have a variable region domain. The term variable region domain as used herein is intended to mean that part of a heavy or light chain which contains the antigen binding site (hereinafter a V_H or V_L domain). The V_H or V_L domain may be of any size or amino acid composition and will generally comprise at least one hypervariable amino acid sequence responsible for antigen binding embedded in a framework sequence.

Each V_H or V_L domain may be any naturally occurring variable domain or an engineered version thereof. By engineered version is meant a variable region domain which has been created using recombinant DNA engineering techniques. Such engineered versions include those created for example from natural antibody variable regions by insertions, deletions or changes in or to the amino acid sequences of the natural antibodies. Particular examples of this type include those engineered V_H or V_L domains containing at least one CDR and optionally one or more framework amino acids from one antibody and the remainder of the variable region domain from a second antibody.

Each V_H domain will generally be covalently attached to at least one cysteine residue. The location of each cysteine residue may be varied according to the size and nature of the antibody fragment required. Thus, in one extreme example a cysteine residue may be attached directly to the C-terminal amino acid of the V_H domain. This may then function as the bridging site for an interchain bridge containing a polymer molecule. Two V_H domains of this type may thus be bridged to form a fragment according to the invention.

In practice however, it is generally preferable that the V_H domain is covalently attached at the C-terminal amino acid to at least one other antibody domain or a fragment thereof which contains a cysteine residue. Thus, for example a V_H domain may be linked to an immunoglobulin C_H1 domain or a fragment thereof. The CH1 domain may be extended with further amino acids, for example to provide a hinge region domain as found generally in immunoglobulins, or to provide further domains, such as antibody CH2 and CH3 domains. In each of the above cases at least one

where the product remains in the circulation it may be advantageous to use a higher molecular weight polymer, for example in the range 25000Da to 40000Da.

In general, each polymer molecule in the antibody fragment according to the invention forms part of an interchain bridge. Each bridge serves to link two heavy chains and in each chain will be covalently linked to a sulphur atom of a cysteine residue. The covalent linkage will generally be a disulphide bond or, in particular a sulphur-carbon bond.

Each interchain bridge may in general be of any desired length or composition. Suitable bridges include residues of homo- or heterofunctional cross-linking reagents, particularly homo- or heterobifunctional cross-linking reagents containing one or more covalently linked polymer molecules as just described.

Homo- or heterofunctional cross-linking reagents include polyvalent, especially bivalent radicals of aliphatic, heteroaliphatic, cycloaliphatic, hetercycloaliphatic, aromatic or heteroaromatic groups containing two thiol reactive functional groups. Each fragment according to the invention will have an interchain bridge derived from such a reagent in which each thiol reactive functional group is in covalent linkage with a sulphur atom of a cysteine residue. Particular thiol reactive functional groups include α-halocarboxylic acids or esters, e.g. iodoacetamide, imides, e.g. maleimide, vinyl sulphones or disulphides.

Particular bridges include optionally substituted straight or branched C_{4-20} alkylene, C_{4-20}alkenylene or C_{4-20}alkynylene chains optionally interrupted by one or more heteroatoms or heteroatom-containing groups such as -O- or -S- atoms or -N(R^1)- [where R^1 is a hydrogen atom or a C_{1-6}alkyl group], -CON(R^1)-, -N(R^1)CO-, -SO_2N(R^1)-, -N(R^1)SO_2-, -C(O)-, -S(O)-, -$S(O)_2$-, -OCON(R^1)-, -N(R^1)C(O)O-, -C(O)O- groups, or by cyclopentylene, cyclohexylene, phenylene or substituted phenylene groups. Optional substituents include for example one or more amino or substituted amino groups, e.g. -N(R^1)$_2$ groups where each R^1 atom or group may be the same or different.

proteins, for example enzymes, nucleic acids and fragments thereof, e.g. DNA, RNA and fragments thereof, radionuclides, particularly radioiodide, and chelated metals. Suitable reporter groups include chelated metals, fluorescent compounds or compounds which may be detected by NMR or ESR spectroscopy.

Particular antineoplastic agents include cytotoxic and cytostatic agents, for example alkylating agents, such as nitrogen mustards (e.g. chlorambucil, melphalan, mechlorethamine, cyclophosphamide, or uracil mustard) and derivatives thereof, triethylenephosphoramide, triethylenethiophosphoramide, busulphan, or cisplatin; antimetabolites, such as methotrexate, fluorouracil, floxuridine, cytarabine, mercaptopurine, thioguanine, fluoroacetic acid or fluorocitric acid, antibiotics, such as bleomycins (e.g. bleomycin sulphate), doxorubicin, daunorubicin, mitomycins (e.g. mitomycin C), actinomycins (e.g. dactinomycin) plicamycin, calichaemicin and derivatives thereof, or esperamicin and derivatives thereof; mitotic inhibitors, such as etoposide, vincristine or vinblastine and derivatives thereof; alkaloids, such as ellipticine; polyols such as taxicin-I or taxicin-II; hormones, such as androgens (e.g. dromostanolone or testolactone), progestins (e.g. megestrol acetate or medroxyprogesterone acetate), estrogens (e.g. dimethylstilbestrol diphosphate, polyestradiol phosphate or estramustine phosphate) or antiestrogens (e.g. tamoxifen); anthraquinones, such as mitoxantrone, ureas, such as hydroxyurea; hydrazines, such as procarbazine; or imidazoles, such as dacarbazine.

Particularly useful effector groups are calichaemicin and derivatives thereof (see for example South African Patent Specifications Nos. 85/8794, 88/8127 and 90/2839).

Chelated metals include chelates of di-or tripositive metals having a coordination number from 2 to 8 inclusive. Particular examples of such metals include technetium (Tc), rhenium (Re), cobalt (Co), copper (Cu), gold (Au), silver (Ag), lead (Pb), bismuth (Bi), indium (In), gallium (Ga), yttrium (Y), terbium (Tb), gadolinium (Gd), and scandium (Sc). In general the metal is preferably a radionuclide. Particular radionuclides include

conventional immunisation and cell fusion procedures], using any suitable standard enzymatic cleavage and/or digestion techniques, for example by treatment with pepsin. Alternatively, the antibody starting material may be prepared by the use of recombinant DNA techniques involving the manipulation and re-expression of DNA encoding antibody variable and/or constant regions. Such DNA is known and/or is readily available from DNA libraries including for example phage-antibody libraries [see Chiswell, D J and McCafferty, J. Tibtech. 10 80-84 (1992)] or where desired can be synthesised. Standard molecular biology and/or chemistry procedures may be used to sequence and manipulate the DNA, for example, to introduce codons to create cysteine residues, to modify, add or delete other amino acids or domains as desired.

From here, one or more replicable expression vectors containing the DNA may be prepared and used to transform an appropriate cell line, e.g. a non-producing myeloma cell line, such as a mouse NSO line or a bacterial, e.g. *E.coli* line, in which production of the antibody fragment will occur. In order to obtain efficient transcription and translation, the DNA sequence in each vector should include appropriate regulatory sequences, particularly a promoter and leader sequence operably linked to the variable domain sequence. Particular methods for producing antibody fragments in this way are generally well known and routinely used. For example, basic molecular biology procedures are described by Maniatis *et al* [Molecular Cloning, Cold Spring Harbor Laboratory, New York, 1989]; DNA sequencing can be performed as described in Sanger *et al* [PNAS 74, 5463, (1977)] and the Amersham International plc sequencing handbook; and site directed mutagenesis can be carried out according to the method of Kramer *et al* [Nucl. Acids Res. 12, 9441, (1984)] and the Anglian Biotechnology Ltd handbook. Additionally, there are numerous publications, including patent specifications, detailing techniques suitable for the preparation of antibodies by manipulation of DNA, creation of expression vectors and transformation of appropriate cells, for example as reviewed by Mountain A and Adair, J R in Biotechnology and Genetic Engineering Reviews [ed. Tombs, M P, 10, Chapter 1, 1992, Intercept, Andover, UK] and in International Patent Specification No. WO 91/09967.

The antibody fragment according to the invention may be useful in the detection or treatment of a number of diseases or disorders. Such diseases or disorders may include those described under the general headings of infectious disease, e.g. viral infection; inflammatory disease/autoimmunity e.g. rheumatoid arthritis, osteoarthritis, inflammatory bowel disease; cancer; allergic/atopic disease e.g. asthma, eczema; congenital disease, e.g. cystic fibrosis, sickle cell anaemia; dermatologic disease, e.g. psoriasis; neurologic disease, e.g. multiple sclerosis; transplants e.g. organ transplant rejection, graft-versus-host disease; and metabolic/idiopathic disease e.g. diabetes.

The antibody fragments according to the invention may be formulated for use in therapy and/or diagnosis and according to a further aspect of the invention we provide a pharmaceutical composition comprising a modified monovalent antibody fragment comprising a monovalent antibody fragment and at least one polymer molecule in covalent linkage characterised in that each covalent linkage is through a sulphur atom of a cysteine residue located in the antibody fragment outside of the variable region domain of the fragment, together with one or more pharmaceutically acceptable excipients, diluents or carriers.

As explained above, the antibody fragment in this aspect of the invention may be optionally linked to one or more effector or reporter groups.

The pharmaceutical composition may take any suitable form for administration, and, preferably is in a form suitable for parenteral administration e.g. by injection or infusion, for example by bolus injection or continuous infusion. Where the composition is for injection of infusion, it may take the form of a suspension, solution or emulsion in an oily or aqueous vehicle and it may contain formulatory agents such as suspending, preservative, stabilising and/or dispersing agents.

Alternatively, the antibody composition may be in dry form, for reconstitution before use with an appropriate sterile liquid.

Figure 3 Inhibition of SK-5 proliferation response to 10ng/ml PDGF BB by anti-PDGFßR DFM-PEG (40kDa random), DFM-PEG (40kDa site-specific), DFM-PEG (10kDa SS linker, site-specific), DFM and IgG.

Figure 4 Inhibition of SK-5 proliferation response to 10ng/ml or 20ng/ml PDGF BB by 1 microgram/ml of anti-PDGFßR DFM-PEG (40kDa random), DFM-PEG (40kDa site-specific), DFM-PEG (10kDa SS linker, site-specific), DFM and IgG.

Figure 5 Pharmacokinetics of ^{125}I-labelled anti-PDGFßR DFM-PEG (10kDa, SS linker) and DFM-PEG (20kDa, SS linker) compared to DFM in rats.

Figure 6 SDS-PAGE analysis under non-reducing (lanes 1-7) and reducing (lanes 8-13) conditions.

Figure 7 Pharmacokinetics of ^{125}I-labelled anti-PDGFßR DFM-PEG (site-specific) of different types compared to unmodified DFM in rats.

Figure 8 SDS-PAGE analysis under non-reducing (lanes 2-3) and reducing (lanes 4-5) conditions.

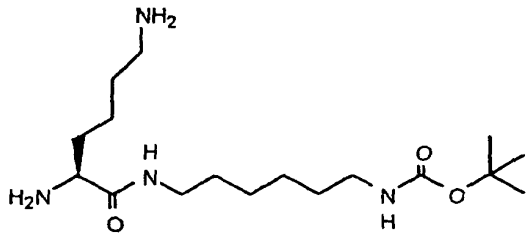
Figure 9 Pharmacokinetics of ^{125}I-labelled hTNF40 DFM-PEG (40kDa, site-specific) compared to DFM and IgG in rats.

The following Examples illustrate the invention.

The following abbreviations are used:

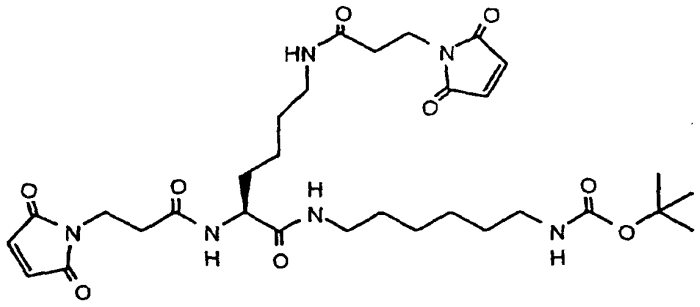
PEG	-	$CH_3O(CH_2CH_2O)_n(CH_2)_2NHCO(CH_2)_2$-
DFM-PEG	-	an antibody fragment according to the invention in which two Fab' fragments are cross-linked with a PEGylated dimaleimide bridge.
DTDP	-	4,4'-dithiodipyridine
AUC	-	area under the curve
RT	-	room temperature
TFA	-	trifluoroacetic acid

INTERMEDIATE 2



Intermediate 1 (26.15g, 42.7mmol) was dissolved (with warming) in ethanol (700ml) and treated with 10% Pd/C (4.2g). The reaction was stirred under H_2 gas in a hot water bath (~30°) for 4h and then allowed to cool. CH_2Cl_2 (20ml) was added and the mixture was filtered and washed well with CH_2Cl_2 and ethanol. Removal of the solvent *in vacuo* gave Intermediate 2 as an oily foam (13.8g, 94%). ^1H NMR ($(CH_3)_2SO$) δ 7.78 (1H, t), 3.06-2.91 (3H, m), 2.89-2.84 (4H, m), 2.52-2.43 (4H, bs) and 1.57-1.15 (23H, m. including 9H, s).

INTERMEDIATE 3



Intermediate 2 (1.47g, 4.47mmol) was dissolved in anhydrous DMF (25ml) and N-succinimidyl 3-maleimidopropionate (22.38g, 8.97mmol) added. The reaction mixture was stirred at room temperature for 3h. The solvent was removed *in vacuo*, and CH_2Cl_2 (50ml) was added followed by saturated $NaHCO_3$ (50ml). The layers were separated and the aqueous layer washed with CH_2Cl_2 (2 x 50ml). The organic layers were combined,

mixture was incubated at RT for 2 hours with occasional agitation. Unreacted PEG was quenched with a 100-fold molar excess of glycine over PEG, added from a stock solution of 1M glycine in 0.1M phosphate buffer, pH6.0 containing 2mM EDTA, and the mixture incubated for a minimum of a further 10 minutes to obtain the desired PEGylated bridging group.

Preparation of Fab'

Fab' from the engineered human antibody g162, which recognises human PDGF β receptor, (hereinafter PDGFβR) was expressed in *E. coli* as described in International Patent Application No. PCT/GB97/03400. The Fab' fragment has a single cysteine residue present in its hinge region available for cross-linking. Cells were harvested from fermentation culture by centrifugation and Fab' extracted by resuspending cells in 100mM tris pH7.4 containing 10mM EDTA and incubating at 60oC overnight. Fab' was then purified by expanded bed chromatography using a column of Streamline ATM (Pharmacia) which was pre-equilibrated with 1M glycine/glycinate pH8.0. The sample was made 1M with respect to glycine and and the pH adjusted to 7.5 with 50% (w/v) sodium glycinate before application to the column in expanded bed mode. After washing with equilibration buffer, the column material was packed into a packed bed and Fab' was eluted with 0.1M citrate pH3.0.

Further purification was achieved by adjusting the pH of the eluate to 7.5 with 2M tris and applying to a column of Protein G sepharose pre-equilibrated with phosphate buffered saline pH7.4. After washing with equilibration buffer, Fab' was eluted with 0.1M glycine-HCl pH2.7. The pH of the eluted Fab' was then adjusted to 6.0 with 2M tris.

Preparation of anti-PDGFßR DFM-PEG (site-specific)

Purified anti-PDGFßR Fab' was diafiltered into 0.1M phosphate buffer, pH6.0 containing 2mM EDTA. The hinge thiol was activated by reduction with ß-mercaptoethylamine. Fab' was incubated with 5mM ß-mercaptoethylamine in 0.1M phosphate buffer, pH6.0 containing 2mM EDTA for 30 minutes at 37°. The sample was then desalted into 0.1M phosphate buffer, pH6.0 containing 2mM EDTA, using Sephadex G-25

1.3 PEG molecules were attached per DFM as quantified by gel-filtration HPLC analysis. The PEG-DFM was purified by cation exchange chromatography using Mono S. Mono S chromatography was carried out using a column equilibrated with 50mM acetate pH 4.5, after application of sample and washing with equilibration buffer, bound material was eluted using a linear gradient of sodium chloride.

Antigen binding analysis by BIAcore

Kinetic analysis to determine the on and off rates for anti-PDGFβR DFM-PEG binding to PDGFβR was performed using a BIACORE 2000 (Biacore AB). The assay involves capture of a mIgG Fc-PDGFβR fusion molecule by an anti-mouse IgG, which is immobilised on the sensor chip surface, followed by an injection of anti- PDGFβR DFM-PEG. Affinipure F(ab')2 fragment of goat anti-mouse Ig, Fc fragment specific (Jackson ImmunoResearch) was immobilised on a Sensor Chip CM5 via amine coupling chemistry to a level of 11500RU. A blank surface was prepared by following the immobilisation procedure but omitting injection of the capturing molecule. HBS buffer (10mM HEPES pH7.4, 0.15M NaCl, 3mM EDTA, 0.005% Surfactant P20, Biacore AB) was used as the running buffer with a flow rate of 10ml/min. An injection of mIgG Fc-PDGFβR from COS cell supernatant was captured by the immobilised anti-mouse IgG to a level between 200-250RU. Anti- PDGFβR DFM-PEG molecules were titrated over the captured mIgG Fc- PDGFβR surface from 2mg/ml to 0.52mg/ml. Surfaces were regenerated by injecting 10ml of 30mM hydrochloric acid. Injections of mIgG Fc- PDGFβ R and each concentration of anti- PDGFβR DFM-PEG were repeated over the blank surface as controls. The sensorgram for each anti- PDGFβR DFM-PEG concentration was corrected with the corresponding sensorgram for the blank surface after deletion of the mIgG Fc- PDGFβR injection and regeneration step. Kinetic parameters were calculated using BIAevaluation 2.1 software.

Results for anti-PDGFβR DFM-PEG, both prepared from PEGylated Intermediate 4 (site-specific) and randomly derivatised anti-PDGFbR DFM-PEG are shown in Table 1. Unmodified DFM prepared with BMH as cross-linker and IgG were used to compare binding parameters. Binding

Table 2 Pharmacokinetic parameters of anti-PDGFβR IgG, DFM and DFM-PEG (40kDa) site-specific.

	t1/2 α (hours)	t1/2 β (hours)	AUC (0-) (%dose x h)	AUC (% of IgG value)
IgG	5.3 +/- 1.3	95.9 +/- 10.9	6442 +/- 525	100
DFM	0.86 +/- 0.1	28.7 +/- 11.6	283 +/- 71	4.4
DFM-PEG (40kDa)	————	33.6+/- 4.5	5318 +/- 1190	82

Bioassay

The potency of anti-PDGFβR DFM and DFM-PEG samples was tested by their ability to block 3H thymidine incorporation by SK-5 dermal fibroblasts in response to PDGF BB. SK-5 cells (grown in DMEM + 10% heat inactivated fetal calf serum, 1% glutamine, !% sodium pyruvate and 0.025 M HEPES buffer) were trypsinised at 80% confluence and seeded at 5,000 cells/0.1 ml per well in a 96 well tissue culture plate, in serum-free media (1:1 DMEM: HAM's F12 + 5 ug/ml insulin, 16 ng/ml selenium, 20 ug/ml transferrin, 1 mg/ml bovine serum albumin, 1% glutamine, 0.025 M HEPES buffer and pen. strep.) Cells were placed in a 37°, 5% CO_2, 95% humidity, incubator for 24h to quiesce. Media alone, antibody alone, PDGF BB at 10 ng/ml or 20 ng/ml final concentration, or PDGF BB together with varying concentrations of antibody were added to the wells, to a final volume of 0.2 ml. Between 5 and 10 wells were used for each condition. 6-8h later, 3H thymidine (0.5 mCi per well) was added and cells left overnight. Plates were removed from the incubator and placed at -20° for 24h in order to facilitate harvesting. Plates were thawed and DNA harvested onto filter mats using a Skatron Micro96 Harvester. Mats were dried at 67° for 90 min, Betaplate Scint (Wallac) added and mats counted in a LKB Wallac 1205 BETAPLATER liquid scintillation counter.

At 10 ng/ml PDGF BB, and 10 mg/ml of anti-PDGFβR DFM or DFM-PEG, incorporation of 3H thymidine is inhibited by 85-92% by all forms of the antibody. As the concentration of antibody is decreased, the difference

These DFM-PEG conjugates were then examined in a pharmacokinetic study in rats using the method described in Example 1. Results (Figure 5) demonstrate significantly slower blood clearance, (longer in vivo half-life) for the DFM-PEG conjugates compared to unmodified DFM.

EXAMPLE 3

Preparation of DFM-PEG conjugates using 5KDa PEG-SCM, 20KDa PEG-SPA, 10K PEG2-NHS and 20K PEG2-NHS derivatives.

Intermediate 4 was derivatised with 20kDa PEG-succinimidyl propionate (Shearwater Polymers Inc.*ibid*), or 5kDa PEG-succinimidyl ester of carboxy-methylated PEG (Shearwater Polymers Inc.), or 10KDa PEG2-succinimide (2 x 5KDa, Polymer Labs) or 20KDa PEG2-succinimide (2 x 10KDa, Polymer Labs), and used to prepare anti-PDGFßR DFM-PEG conjugates as described in Examples 1 & 2. These DFM-PEG derivatives were purified using ion-exchange chromatography followed by gel filtration as described in Example 2. SDS-PAGE analysis revealed that conjugation to PEG was successful in all cases. (Figure 6). BIAcore analysis was also carried out to determine antigen binding affinity. Results shown in Table 4 demonstrate that these PEG derivatives can be attached with little loss in antigen binding affinity.

Table 4 BIAcore analysis of DFM-PEG derivatives

Sample	kass	kdiss	Kd (M)
DFM	1.81×10^{7}	2.29×10^{-3}	1.27×10^{-10}
DFM-PEG 5kDa (SCM linkage)	1.89×10^{7}	2.58×10^{-3}	1.37×10^{-10}
DFM-PEG 20kDa (SPA linkage)	9.80×10^{6}	2.37×10^{-3}	2.42×10^{-10}
DFM-PEG 10kDA (5kDA x 2, NHS linkage)	1.56×10^{7}	2.14×10^{-3}	1.38×10^{-10}
DFM-PEG2 20kDa (10kDa x 2, NHS linkage)	1.11×10^{7}	2.20×10^{-3}	1.99×10^{-10}

with equilibration buffer, bound material was eluted with a linear gradient of sodium chloride. Purified material was examined on SDS-PAGE and shown to have a slower mobility than unmodified DFM demonstrating successful conjugation of PEG (Figure 8).

Antigen binding analysis by BIAcore

Antigen binding activity was assessed by BIAcore assay which measured affinity for TNF binding. Fab', DFM, IgG or PEG-DFM were captured with an immobilized anti-Fab' antibody, and human TNF passed over the surface. The kinetics of TNF binding were then analysed. Results from this analysis are shown in Table 5. Unmodified DFM prepared with BMH as cross-linker and IgG were used to compare binding parameters. Binding affinity as quantified by the Kd value was similar between IgG and DFM (1.79×10^{-10}M and 1.07×10^{-10}M. respectively). Site-specific PEGylation of DFM resulted in an almost identical binding affinity of 1.82×10^{-10}M, suggesting no loss of antigen binding function after PEG modification.

Table 5: BIAcore analysis of DFM-PEG 40kDa compared to IgG, Fab' and unmodified DFM.

Sample	kass	kdiss	Kd (M)
IgG	4.21×10^{5}	7.54×10^{-5}	1.79×10^{-10}
DFM	2.97×10^{5}	3.17×10^{-5}	1.07×10^{-10}
DFM-PEG 40kDa (site-specific)	3.31×10^{5}	6.02×10^{-5}	1.82×10^{-10}

Pharmacokinetics

For pharmacokinetic analysis, these samples were studied in rats using the method described in Example 1. The clearance rates and Auc values

CLAIMS

1. A divalent antibody fragment comprising two antibody heavy chains and at least one polymer molecule in covalent linkage, each heavy chain being covalently linked to the other by at least one non-disulphide interchain bridge linking the sulphur atom of a cysteine residue in one chain to the sulphur atom of a cysteine residue in the other chain, said cysteine residues being located outside of the variable region domain of each chain, characterised in that at least one non-disulphide interchain bridge contains a covalently linked polymer molecule.

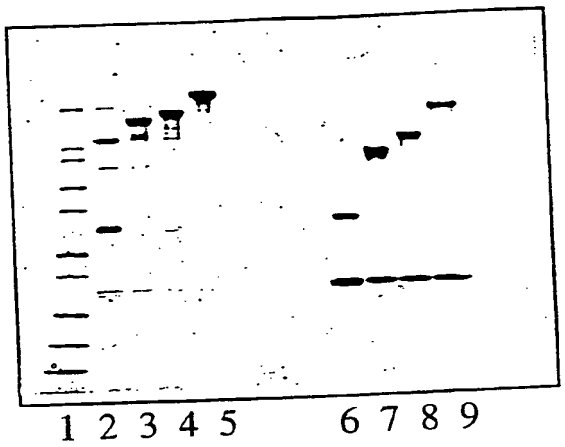
2. An antibody fragment according to Claim 1 in which each heavy chain is covalently linked to the other by a single non-disulphide bridge, said bridge containing a covalently linked polymer moelcule.

3. An antibody fragment according to Claim 1 or Claim 2 wherein each heavy chain is paired with a light chain.

4. An antibody fragment according to any one of Claims 1 to Claim 3 wherein each heavy chain is a V_H-CH1 chain terminally substituted by a hinge region domain.

5. An antibody fragment according to Claim 4 wherein each non-disulphide bridge present links the sulphur atom of a cysteine residue located in the hinge region domain of one heavy chain, to the sulphur atom of a cysteine residue in the hinge region domain of the other chain.

6. An antibody fragment according to any one of Claim 1 to Claim 5 wherein the polymer is an optionally substitued straight or branched chain polyalkylene, polyalkenylene or polyoxyalkylene polymer or a branched or unbranched polysaccharide.



Non-Reducing

1. Molecular weight marker proteins
2. Fab' and DFM mix
3. Purified DFM-PEG (10kDa SS linker)
4. Purified DFM-PEG (20kDa SS linker)
5. Purified DFM-PEG (40kDa)

Reducing

6. Fab' and DFM mix
7. Purified DFM-PEG (10kDa SS linker)
8. Purified DFM-PEG (20kDa SS linker)
9. Purified DFM-PEG (40kDa)

FIGURE 1

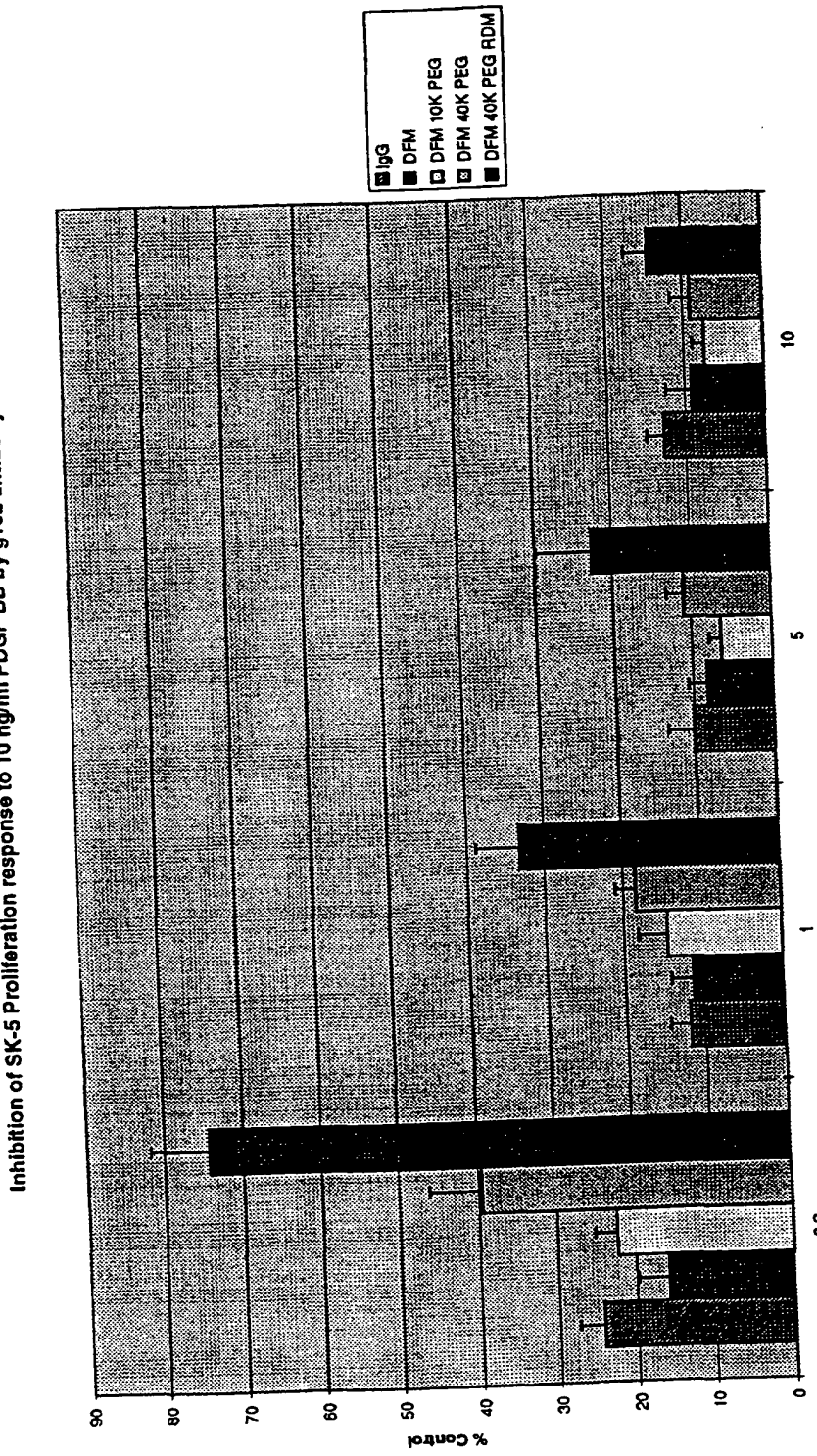

Inhibition of SK-5 Proliferation response to 10 ng/ml PDGF BB by g162 antibody variants
IgG
DFM
DFM 10K PEG
DFM 40K PEG
DFM 40K PEG RDM
% Control
0
10
20
30
40
50
60
70
80
90
0.2
1
5
10
ug/ml Mab

FIGURE 3

ECE29 & 31 : Pharmacokinetics of 125-I anti-PDGF-R DFM in rats

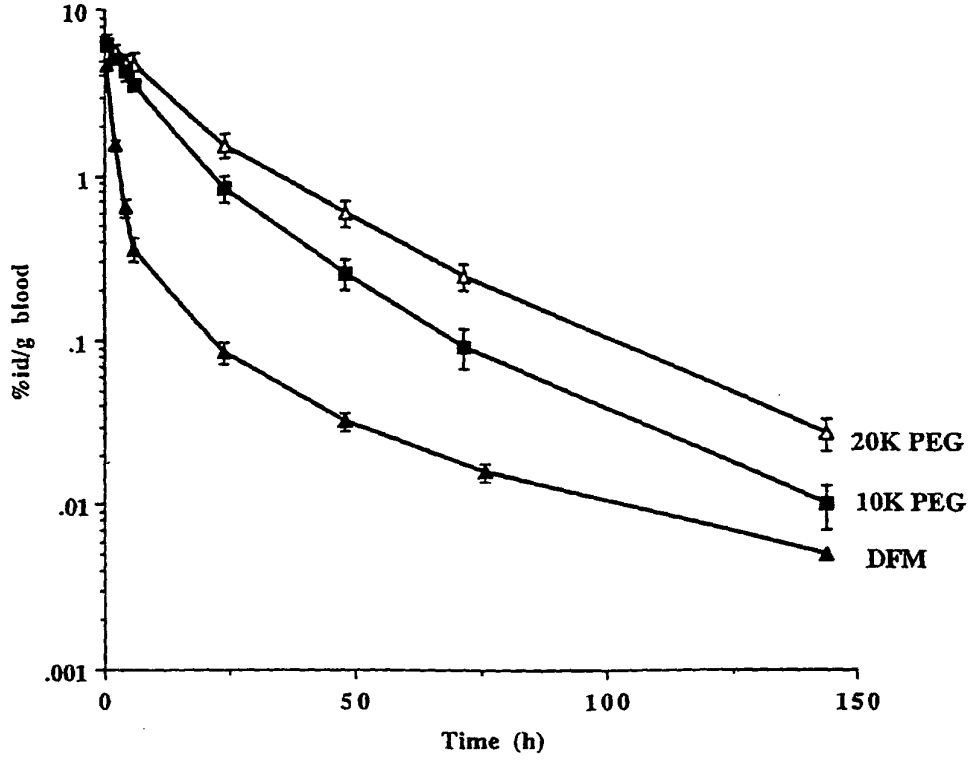
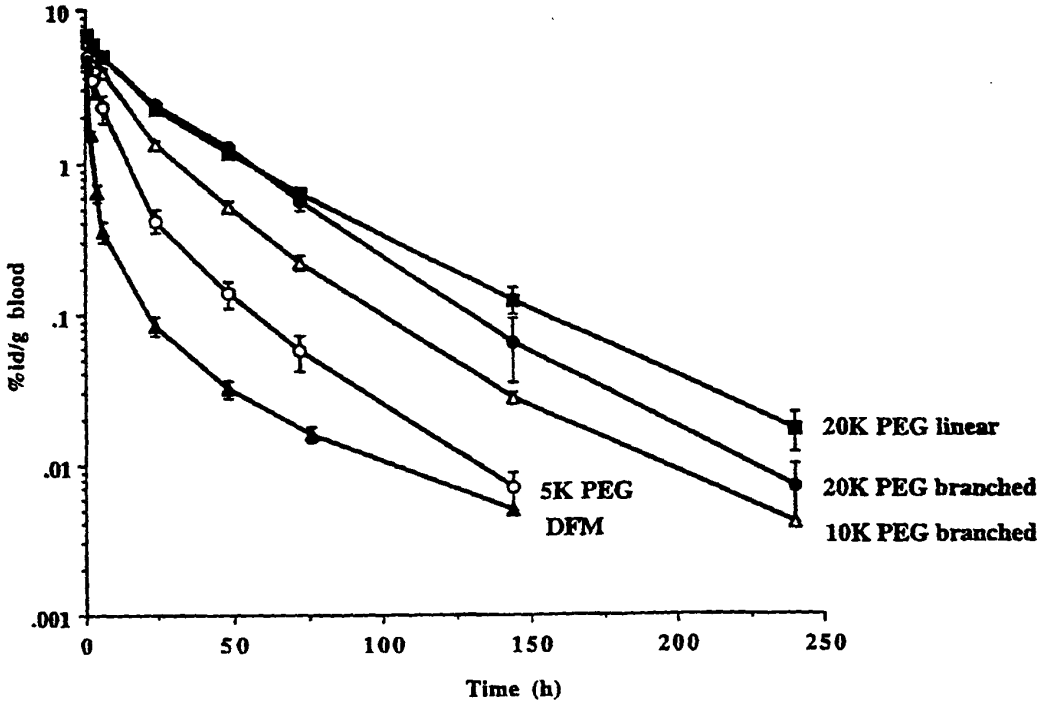


FIGURE 5

ECE29 & 32 : Pharmacokinetics of 125-I anti-PDGF-R DFM in rats



FIUGURE 7

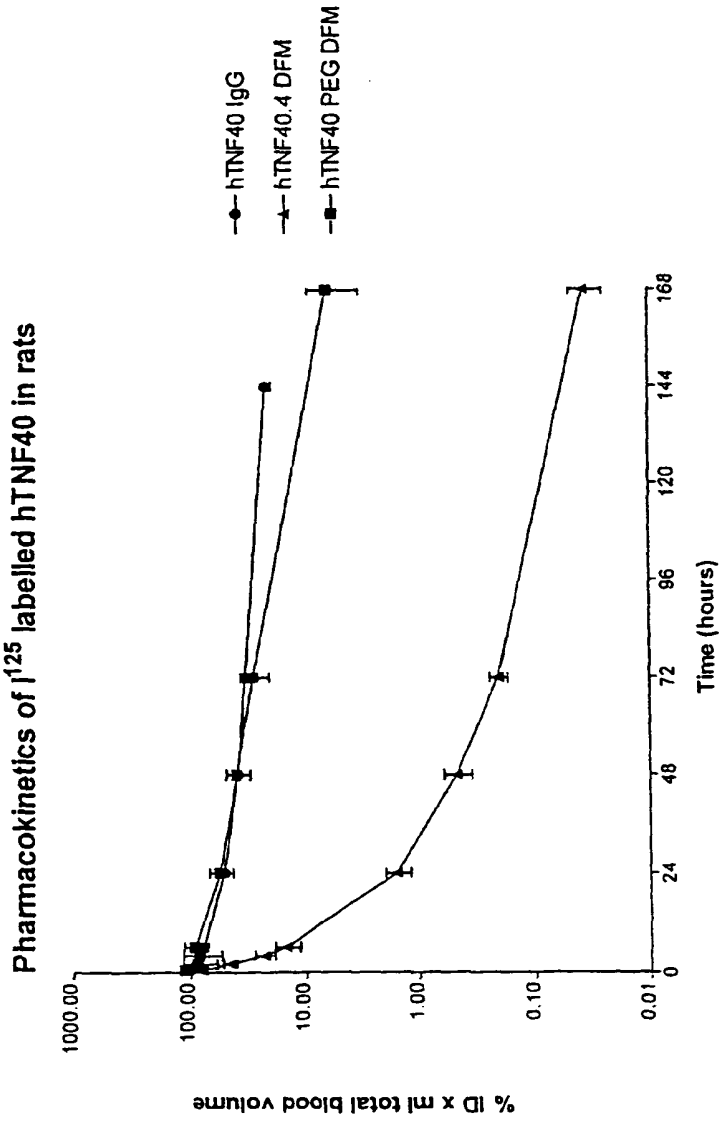

Pharmacokinetics of I125 labelled hTNF40 in rats
hTNF40 IgG
hTNF40.4 DFM
hTNF40 PEG DFM
% ID x ml total blood volume
1000.00
100.00
10.00
1.00
0.10
0.01
0
24
48
72
96
120
144
168
Time (hours)

FIGURE 9